30. Lindsley, D. L. & Zimm, G. G. *The Genome of* Drosophila melanogaster (Academic, San Diego, 1992).
31. Motzny, C. K. & Holmgren, R. The *Drosophila* cubitus interruptus protein and its role in the wingless and hedgehog signal transduction pathways. *Mech. Dev.* **52,** 137–150 (1995).

Acknowledgements. We thank J. Nüsslein-Volhard, Y. Chen and G. Struhl for fly stocks; R. Burke, C. Dahmann, N. Méthot and particularly G. Struhl for comments on the manuscript; F. Cifuentes, D. Nellen and M. Zecca for help with the clonal analysis; and J. Modolell for support. I.R. was the recipient of an EMBO fellowship. This work was supported by the Swiss National Science Foundation, the Direccion general de Investigacion Cientifica y Tecnica, and the Fundacion Ramon Areces.

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Opposing BMP and EGF signalling pathways converge on the TGF-β family mediator Smad1

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The growth factor TGF-β, bone morphogenetic proteins (BMPs) and related factors regulate cell proliferation, differentiation and apoptosis, controlling the development and maintenance of most tissues[1,2]. Their signals are transmitted through the phosphorylation of the tumour-suppressor SMAD proteins by receptor protein serine/threonine kinases (RS/TKs)[3–10], leading to the nuclear accumulation[5,9,11,12] and transcriptional activity of SMAD proteins[6,12,13]. Here we report that Smad1, which mediates BMP signals, is also a target of mitogenic growth-factor signalling through epidermal growth factor and hepatocyte growth factor receptor protein tyrosine kinases (RTKs). Phosphorylation occurs at specific serines within the region linking the inhibitory and effector domains of Smad1, and is catalysed by the Erk family of mitogen-activated protein kinases. In contrast to the BMP-stimulated phosphorylation of Smad1, which affects carboxy-terminal serines and induces nuclear accumulation of Smad1[6], Erk-mediated phosphorylation specifically inhibits the nuclear accumulation of Smad1. Thus, Smad1 receives opposing regulatory inputs through RTKs and RS/TKs, and it is this balance that determines the level of Smad1 activity in the nucleus, and so possibly the role of Smad1 in the control of cell fate.

Members of the transforming growth factor (TGF)-β family participate in signalling networks that control cell fate in virtually all tissues. Signalling through these networks involves the synergistic or antagonistic interplay of different pathways. One example is the ability of tyrosine kinase receptor activators and BMPs to oppose each others' actions during organ development. Fibroblast growth factor (FGF) opposes the antiproliferative effect of Bmp-2 during limb bud outgrowth[14] and the ability of Bmp-4 to induce interdigital membrane apoptosis during digit formation[15], whereas epidermal growth factor (EGF) can oppose the induction by Bmp-2 of osteogenic differentiation markers[16]. Bmp-2 and Bmp-4 can both counteract the induction by FGF of genes essential for tooth development[17]. The mechanisms underlying these antagonistic interactions are unknown. However, the discovery[3,4] of SMAD proteins as mediators of TGF-β family signalling provides a basis to investigate this problem, especially the anti-BMP effects of tyrosine kinase receptor agonists.

SMAD proteins consist of conserved amino- and carboxy-terminal domains (N and C domains, respectively) linked by a more divergent region. The C domain has an effector function that becomes apparent when the isolated domain is tested in transcriptional assays[12] and mesoderm induction assays[11]. The N domain interacts physically with the C domain[18], inhibiting its effector activity[11,12,18], and contributes to DNA binding[19]. Receptor-mediated phosphorylation of serine residues at the end of the C domain (Fig. 1b) relieves it from the inhibitory action of the N domain[6,9]. Smad1 is activated in this fashion by Bmp-2 and Bmp-4 receptors[5,6], and Smad2 and Smad3 are activated by TGF-β and activin receptors[7,9,20]. Receptor-mediated phosphorylation allows these SMAD proteins to associate with the related Smad4 protein[6,7,21], move into the nucleus[5,6,9,12], and activate gene responses[6,7,9,12,13,20]. Smad4 is the product of the tumour-suppressor gene *deleted in pancreatic carcinoma-4* (*DPC4*)[22], and is a trimeric protein[23] that is required for signalling by different members of the TGF-β family[7,24].

We have previously shown that Smad1 is phosphorylated in proliferating mink lung epithelial cells (R-1B/L17), even in the absence of BMP signals[6]. To investigate the nature of this basal phosphorylation we isolated Smad1 from transiently transfected and ^{32}P-phosphate-labelled R-1B/L17 cells via a Flag epitope and subjected the protein to proteolytic digestion by various enzymes. Separation by SDS–PAGE revealed ^{32}P-phosphate-labelled fragments of relative molecular mass 15,000 and 17,000 (M_r 15K and 17K) in the digests with trypsin and endopeptidase-LysC, respectively, whereas no fragment larger than 3K could be detected in a chymotryptic digest (Fig. 1a). The predicted digestion pattern of Smad1 allowed us to identify the phosphorylated region as part of the linker region of Smad1 that connects the highly conserved N and C domains (Fig. 1b). This identification was confirmed by microsequencing of the ^{32}P-phosphate-labelled tryptic fragment purified by high-performance liquid chromatography (HPLC). The C terminus of Smad1, which is phosphorylated directly by the BMP type I receptor at the SSVS sequence[6] (Fig. 1b), is cleaved by each of these enzymes into a peptide of 2K or less (Fig. 2c).

Smad1 contains predominantly phosphoserine, with some phosphothreonine but not phosphotyrosine[5] (data not shown). To determine the exact sites of phosphorylation in the linker region, almost all serines and threonines in this region were mutated, either singly or in combination, as substitutions of serine to alanine or threonine to valine (Fig. 1b). Analysis of these mutants in transfected cells demonstrated that none of the mutations had a strong effect on BMP-induced phosphorylation (Fig. 1c, and data not shown). However, the mutant Smad1(4SP/AP), which has mutations in four repeated PXSP motifs, showed a strong reduction in basal phosphorylation (Fig. 1c). Mutation of fewer than four PXSP motifs caused a proportional reduction of phosphorylation (data not shown). When the PXSP motifs in the linker region and the BMP receptor target motif SSVS at the C terminus were mutated simultaneously, phosphorylation of Smad1 was completely lost, both in the presence and absence of stimulation by BMPs (Fig. 1d). This indicates that the PXSP motifs are the linker phosphorylation sites, that they are phosphorylated independently of the C-terminal SSVS motif phosphorylation, and that their phosphorylation is mediated by a kinase other than the BMP receptor.

PXSP motifs are consensus sites for mitogen-activated protein kinases (MAP kinases)[25]. Because basal phosphorylation of these sites in Smad1 was observed in cells proliferating in the presence of serum, we investigated whether this phosphorylation was induced by specific growth factors that are known to activate MAP kinases. EGF signals through a receptor tyrosine kinase (RTK) and strongly activates the Erk subfamily of MAP kinases, whereas tumour necrosis factor (TNF)-α, an inflammatory cytokine, signals through a different family of receptors and stimulates the stress-activated MAP kinases Jnk and p38 (ref. 26). In cells transfected with Flag–Smad1 and labelled with ^{32}P-phosphate, EGF induced a rapid increase in Smad1 phosphorylation, whereas TNF-α had no effect (Fig. 2a). Furthermore, mutation of the four PXSP motifs not only reduced the basal phosphorylation of Smad1 in proliferating cells (Fig. 2b, lane 4; see also Fig. 1a, b), but also abolished EGF-induced

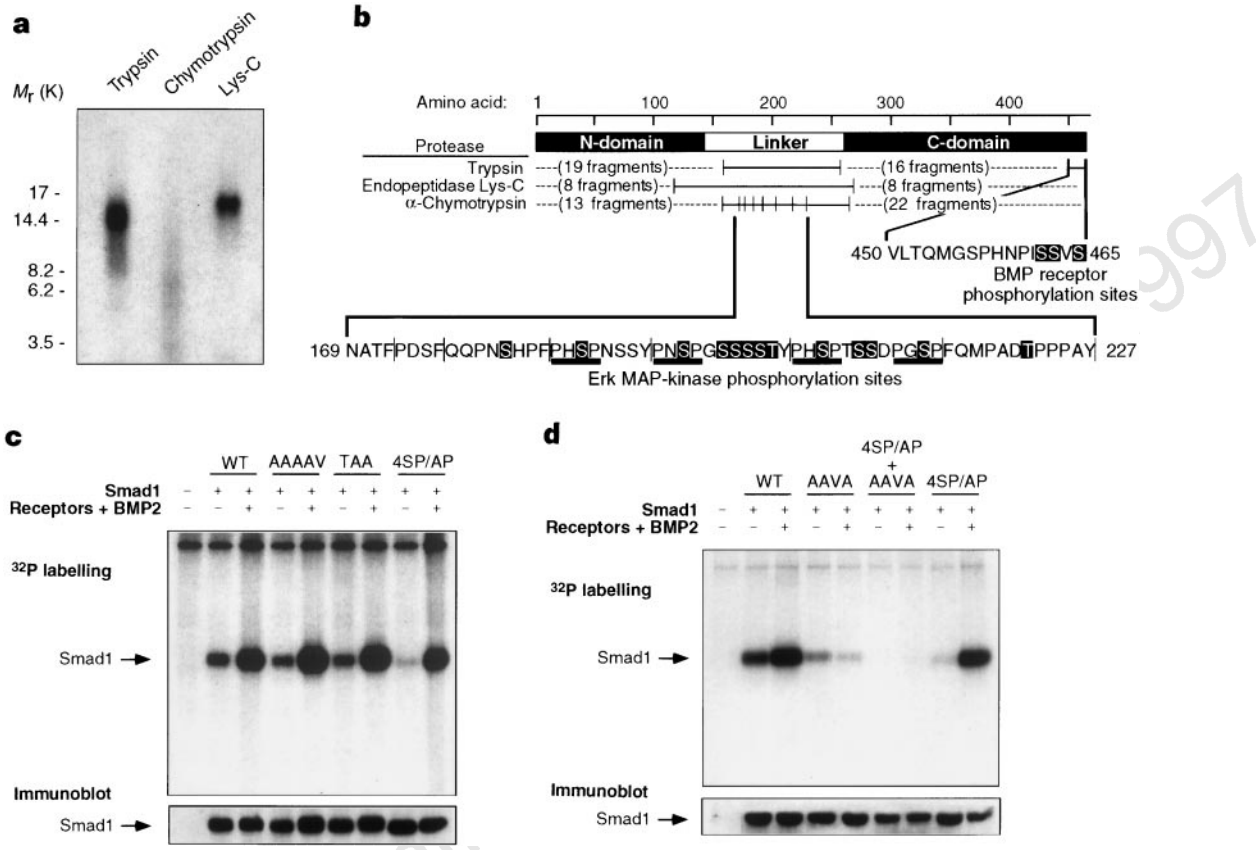


Figure 1 Smad1 is phosphorylated in the linker domain *in vivo*. **a**, Phosphorylated proteolytic fragments correspond by size to the linker region. Fragments were obtained by protease digestion of Smad1 from [^{32}P]-labelled transfected R-1B/L17 cells. **b**, Predicted proteolytic digestion patterns of Smad1 (ref. 12). The partial sequence of the linker region containing potential phosphorylation sites is shown. Residues mutated singly or in groups are highlighted, and Erk MAP kinase consensus sites are underlined. The sequence of the C-terminal tryptic peptide is also listed with the BMP receptor phosphorylation sites highlighted. **c**, **d**, Basal and BMP-induced phosphorylation of the wild-type (WT) Smad1 and selected mutants in transfected R-1B/L17 cells. BMP stimulation was provided by co-transfection of BMP receptors followed by Bmp-2 addition 20 min before cell lysis. Smad1 expression was controlled by anti-Flag immunoblotting. The Smad1 mutants shown contain mutations of S to A or T to V in amino acids 198–202 (AAAAV), 209–210 (TAA), all four PXSP motifs (serines 187, 195, 206 and 214) (4SP/AP), the C-terminal serines (AAVA), or both the PXSP motifs and the C-terminal serines (4SP/AP + AAVA).

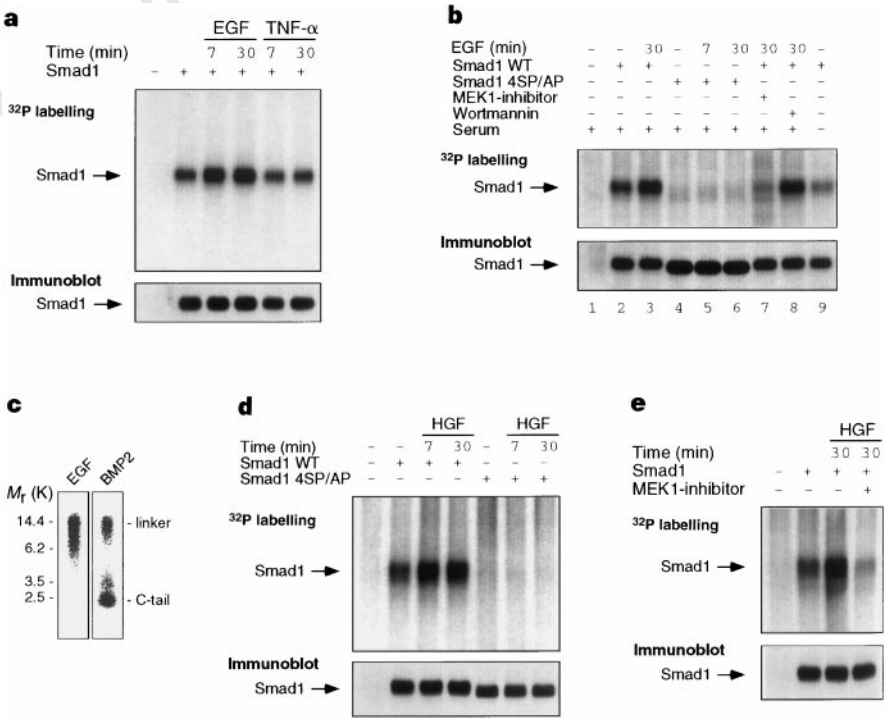


Figure 2 Smad1 is phosphorylated by Erk MAP kinase in response to EGF or HGF. **a**, The effect of EGF and TNF-α on Smad1 phosphorylation in transfected R-1B/L17 cells. [^{32}P]-labelled cells were incubated with growth factors for the times indicated. **b**, **d**, **e**, Effect of inhibitors and Smad1 PXSP site mutations on Smad1 phosphorylation in response to EGF (**b**) or HGF (**d**, **e**). Inhibitors were added 1 h before growth-factor stimulation. Lane 9 shows the effect of 14 h incubation in serum-free medium. **c**, Trypsin digests of [^{32}P]-labelled Flag–Smad1 from EGF-treated or Bmp-2-treated cells showing phosphorylation of the linker region but not the C-terminal tail in EGF-treated cells. Phosphorylation of the linker in Bmp-2-treated cells is attributed to the effect of serum factors in the medium.

Smad1 phosphorylation (Fig. 2b, lanes 5 and 6). Addition of EGF did not lead to phosphorylation of the C-terminal sites in Smad1 (Fig. 2c).

Two signalling pathways that mediate the effects of receptor tyrosine kinases are the Ras pathway, which leads to the activation of Erk MAP kinases through Raf and MEK1 (ref. 27), and the phosphatidylinositol-3-OH kinase (PI(3) kinase) pathway, which activates the $p70^{S6K}$ and c-Akt kinases[28]. Both the basal phosphorylation and the EGF-stimulated phosphorylation of Smad1 were strongly suppressed by a specific inhibitor of MEK1, whereas wortmannin, an inhibitor of PI(3) kinase, had no effect (Fig. 2b, lanes 7 and 8). To determine whether Smad1 phosphorylation is induced by other growth factors that activate the MAP kinase pathway, we performed similar experiments using hepatocyte growth factor (HGF). HGF is a potent mitogen for epithelial and endothelial cells, signals through the RTK c-Met, and also activates Erk MAP kinases[29]. Like EGF, HGF rapidly induced Smad1 phosphorylation, and this induction was abolished by mutation of the

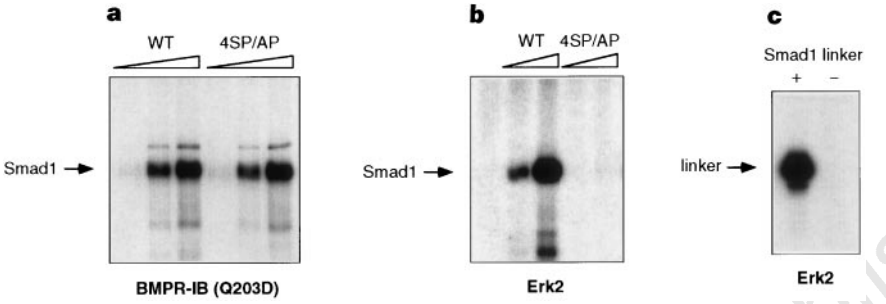


Figure 3 Smad1 is phosphorylated by Erk2 at PXSP sites *in vitro*. **a**, **b**, Purified, recombinant Smad1 proteins (wild-type (WT) or the quadruple PXSP mutant (4SP/AP)) were tested at concentrations in the nanomolar range as substrates of recombinant activated BMP type I receptor kinase (BMPR-IB (Q203D)) or recombinant activated Erk2. **c**, A purified, recombinant Smad1 linker domain was tested as substrate for Erk2.

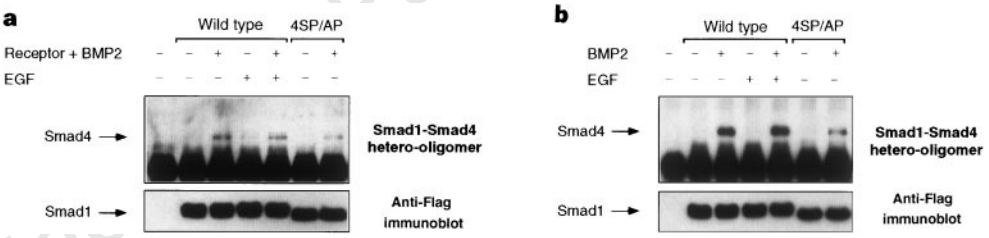
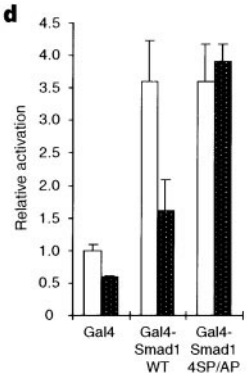
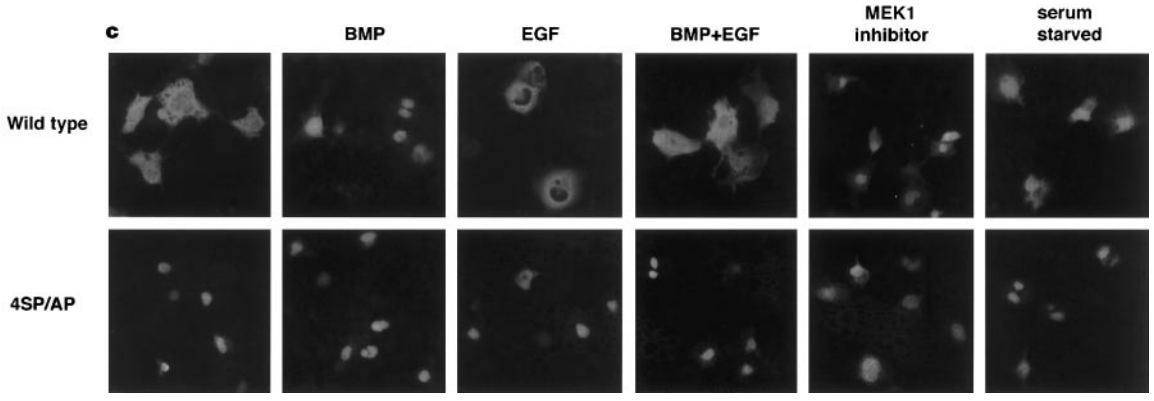


Figure 4 Phosphorylation in the linker region regulates cellular localization and transcriptional function of Smad1 but not association with Smad4. **a**, COS-1 cells were transiently transfected with haemagglutinin-tagged Smad4, and with Flag-tagged Smad1 (wild-type or 4SP/AP mutant) and BMP type I receptor (BMPR-IB (Q203)) as indicated. Cells were treated with EGF for 15 min before Bmp-2 addition. Then, 1 h after Bmp-2 addition, cells were lysed and subjected to anti-Flag immunoprecipitation followed by anti-haemagglutinin immunoblotting. Smad1 expression levels were controlled by anti-Flag immunoblotting of the same cell lysates. **b**, A similar experiment was performed without transfection of the BMP type I receptor. **c**, COS-1 cells were transiently transfected with Flag-tagged wild-type Smad1 or the mutant Smad1 (4SP/AP). Where indicated, cells were treated with EGF 15 min before Bmp-2 addition. Then, 30 min after Bmp-2 addition, immunostaining was performed using anti-Flag monoclonal antibody and FITC-conjugated secondary antibody. Treatment with MEK1 inhibitor and serum starvation was for 1 h and for 16 h, respectively, before immunostaining. The same slides were counterstained with DAPI to visualize nuclei (data not shown). Several independent experiments were performed with essentially identical results. **d**, R-1B/L17 cells transfected with a Gal4-dependent Luciferase reporter construct and the indicated Gal4 expression constructs were serum starved for 12 h and then treated with or without Bmp-2 in the absence (white bars) or presence (black bars) of EGF. Luciferase activity was measured 18 h after factor addition and plotted as relative activation by Bmp-2. Experiments were performed in triplicate.





four PXSP motifs (Fig. 2c) or the addition of MEK1 inhibitor (Fig. 2d). Taken together, these results provide strong evidence that Smad1 is indeed a target of the Erk subfamily of MAP kinases in the cell. Consistent with this, the basal phosphorylation of Smad1 is reduced upon serum starvation (Fig. 2b, lane 9), a condition that decreases Erk activity. The residual phosphorylation is probably caused by low levels of Erk activity, which remains even after serum starvation.

To provide evidence that Erk kinases can phosphorylate Smad1 directly at the identified sites, we used bacterially expressed, highly purified Smad1 and the mutant Smad1(4SP/AP) as substrates in *in vitro* kinase assays. Both forms of Smad1 were equally well phosphorylated when titrated in kinase reactions with activated BMP type I receptor kinase (Fig. 3a). This is consistent with the observations that the BMP receptor kinase phosphorylates Smad1 at the C-terminal serines[6] and that the linker mutations do not affect BMP-induced phosphorylation of Smad1 *in vivo* (Fig. 1c, d). Titration of wild-type Smad1 in kinase reactions with activated Erk2 demonstrated that wild-type Smad1 is a substrate for Erk2 at nanomolar concentrations *in vitro* (Fig. 3b). In contrast to the BMP receptor kinase, Erk2 failed to phosphorylate Smad1(4SP/AP) (Fig. 3b). Furthermore, the isolated recombinant linker region of Smad1 was also phosphorylated by Erk2 (Fig. 3c). Thus Erk2 can potently phosphorylate Smad1 *in vitro* at the same sites that are phosphorylated in response to EGF or HGF *in vivo*.

To understand the functional significance of Smad1 phosphorylation by MAP kinases, we investigated the potential effects on Smad1 activation events. We compared wild-type Smad1 and the mutant Smad1(4SP/AP) for their ability to associate with Smad4/DPC4 in response to BMP signalling[6,7]. In cells co-transfected with BMP receptors and epitope-tagged Smad1 and Smad4, Smad1 association with Smad4 in response to Bmp-2 was not affected by prior treatment with EGF (Fig. 4a). Even under conditions in which the BMP response was mediated solely by the endogenous BMP receptors, EGF did not significantly alter the association with Smad4 (Fig. 4b). Mutation of the PXSP motifs of Smad1 caused a slight decrease in the BMP-induced association with Smad4 in cells co-transfected with BMP receptors (Fig. 4a), and had a more pronounced effect when the BMP response was mediated solely by endogenous BMP receptors (Fig. 4b). This reduction in BMP-induced association with Smad4 might reflect a decreased access of Smad1(4SP/AP) to the receptor as a result of a preferential localization of this mutant in the nucleus (see below).

In contrast to the relatively weak effects on the association between Smad1 and Smad4, both addition of EGF and the mutations of the PXSP site had striking effects on the subcellular localization of Smad1. Immunofluorescence of Smad1-transfected cells revealed a distribution throughout the cell under basal conditions and a predominantly nuclear localization after Bmp-2 stimulation (Fig. 4c), as previously reported[5,6,12]. The mutant Smad1(4SP/AP) was predominantly nuclear even in the absence of BMP stimulation (Fig. 4c). Treating the cells with EGF caused exclusion of wild-type Smad1 from the nucleus and inhibited BMP-induced nuclear accumulation of wild-type Smad1 (Fig. 4c), but had no effect on the mutant Smad1 (Fig. 4c). Furthermore, addition of the MEK1 inhibitor or serum starvation affected wild-type Smad1 in an opposite way to EGF treatment, leading to nuclear accumulation even in the absence of treatment with BMP (Fig. 4c). MEK1 inhibitor or serum starvation had no effect on the mutant Smad1 (Fig. 4c). These results suggest that Erk-mediated phosphorylation of the linker region inhibits nuclear accumulation of Smad1 and is capable of preventing BMP-induced nuclear accumulation without interfering with formation of the Smad1–Smad4 complex. By using Smad4-defective cells we confirmed that Smad1 association with Smad4 and Smad1 nuclear translocation are independent events (F. Liu, C. Pouponnot and J.M., unpublished data). Because activated Erk kinases are distributed through the cytoplasm and the nucleus[30], phosphorylation of Smad1 by Erk could occur in either compartment and regulate Smad1 recognition by either nuclear import or export mechanisms.

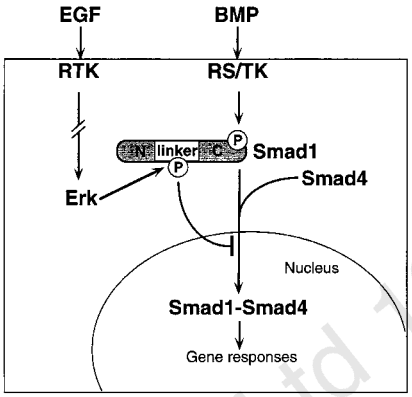


Figure 5 Schematic representation of the regulation of Smad proteins by opposing signalling inputs mediated by receptor tyrosine kinases (RTKs) and receptor serine/threonine kinases (RS/TKs).

To assess the functional consequences of Smad1 regulation by Erk MAP kinases, we sought to analyse the effects of EGF treatment on the transcriptional activity of Smad1 in response to BMP. Because a Smad1-dependent early response reporter gene for BMP in mammalian cells has not yet been described, we resorted to the previously used Gal4-dependent reporter assay[6,12]. Cells expressing a fusion of the Gal4 DNA-binding domain and Smad1 (Gal4–Smad1) showed a BMP-inducible increase in Luciferase activity when co-transfected with a Gal4-dependent Luciferase reporter construct (Fig. 4d). BMP had no effect on the Gal4 DNA-binding domain alone (Fig. 4d). The Gal4–Smad1(4SP/AP) mutant construct was induced by BMP to the same extent as Gal4–Smad1 (Fig. 4d), consistent with the observation that the mutant Smad1 is phosphorylated to a similar extent to wild-type Smad1 in response to BMP (Fig. 1c, d). Most importantly, simultaneous treatment of cells with EGF strongly reduced the BMP-induced transcriptional activity of Gal4–Smad1, whereas EGF had no effect on the activity of Gal4–Smad1(4SP/AP) (Fig. 4d). This suggests that Erk-mediated phosphorylation of Smad1 inhibits BMP-induced nuclear accumulation, and consequently inhibits nuclear functions of Smad1, such as transcriptional activity.

Our data show that Smad1, a mediator of BMP receptor signals, is a target of growth-factor signalling through the Erk MAP kinase pathway (Fig. 5). Erk phosphorylates Smad1 at the linker region preventing nuclear accumulation of Smad1 under basal conditions and inhibiting nuclear accumulation and the transcriptional activity of Smad1 in response to BMP. Erk activators should therefore oppose the functions of BMP that depend on nuclear accumulation and transcriptional activity of Smad1. The differential regulation of Smad1 through RTKs and RS/TKs provides a mechanism for antagonistic actions of mitogenic factors and BMPs. This mechanism may underie, at least in part, the opposing effects of these factors during vertebrate development[14–17]. Smad2 and Smad3, which are TGF-β/activin mediators, contain similar potential phosphorylation sites in their linker region, and may therefore also be targets of proline-directed kinases (M.K. and J.M., unpublished observations). Therefore SMAD regulation by MAP kinases might be a general phenomenon controlling the signalling activity of the TGF-β family. □

Methods

Transfection, metabolic labelling and immunofluorescence assays. All of these procedures were performed essentially as described[6]. In brief, R-1B/L17 cells were transiently co-transfected with Flag-tagged Smad1 (ref. 12) and the BMP receptors BMPR-IB and BMPR-II[12,31] as indicated. All constructs were in pCMV5. Smad1 mutant constructs were obtained by standard *in vitro* muta-

genesis procedures. Cells were metabolically labelled 3 days after transfection with [^{32}P] phosphate for 3 h, treated with the indicated factors (5 nM Bmp-2, Genetics Institute; 18 nM EGF, R&D Systems; 1.1 nM HGF, R&D Systems) and lysed. Where indicated, cells were treated with the MEK1 inhibitor PD98059 (100 μM, New England Biolabs) or the PI(3) kinase inhibitor wortmannin (0.1 μM, Calbiochem) for 1 h before addition of growth factor. Serum starvation was for 14 h before metabolic labelling. After cell lysis, Flag–Smad1 was precipitated with monoclonal anti-Flag antibody (M2, Kodak Scientific) and proteins were resolved by SDS–PAGE and visualized by autoradiography. Parallel cultures were treated equivalently, lysed and subjected to western immunoblotting with anti-Flag antibody M2 and by chemiluminescence (ECL, Amersham).

Kinase assays. Smad1 and the Smad1 linker domain (amino acids 146–264) were subcloned into a pET expression vector (Novagen) encoding an N-terminal hexahistidine tag. Bacterial expression and purification of recombinant proteins were performed as described[6]. Smad1 proteins were preincubated (30 min at 4 °C) with recombinant, activated BMPR-IB(Q203D) cytoplasmic domain (amino acids 150–502)[6] or with recombinant, activated Erk2 MAP kinase (New England Biolabs) in a buffer containing 50 mM Tris-HCl, pH 7.3, 100 mM NaCl, 10 mM $MnCl_2$, 10% (v/v) glycerol, 5 mM dithiothreitol, and 0.05% (v/v) Triton X-100. Upon [γ^{32}P]ATP addition, reactions were performed at 28 °C for 20 min. Reactions were stopped by addition of a buffer containing 6 M guanidinium-HCl, and Smad1 was recovered with Ni-NTA agarose.

Phosphopeptide analysis. Smad1 protein was immunoprecipitated from transfected, [^{32}P]phosphate-labelled R-1B/L17 cells, separated by SDS–PAGE, and transferred to nitrocellulose. Membrane pieces containing Smad1 were incubated with trypsin, chymotrypsin and endopeptidase Lys-C (Promega, Boehringer-Mannheim, and Worthington, respectively) in 50 mM ammonium bicarbonate at 37 °C for 3 h. Digests were resolved on 16.5% Tris-Trycine gels[32] and visualized by autoradiography. M_r markers were ^{14}C-methylated peptide fragments (Sigma).

SMAD association assay. In SMAD association experiments[6], COS-1 cells co-transfected with haemagglutinin-tagged Smad4, Flag-tagged Smad1 and BMPR-IB(Q203D) were treated with EGF (18 nM) for 15 min before Bmp-2 addition. Cells were lysed[6] and portions used in anti-Flag immunoblotting assays to control the Smad1 expression level. The remainder of the cell lysates were immunoprecipitated with anti-Flag antibody. Immunoprecipitates were washed and subjected to anti-haemagglutinin immunoblotting.

Luciferase assays. Luciferase assays were performed essentially as described[7]. In brief, R-1B/L17 cells were transiently cotransfected with a Gal4-luciferase reporter construct[33] (2 μg) and the indicated Gal4 or Gal4-Smad1 expression constructs (0.5 μg). Cells were serum starved for 12 h before treatment with Bmp-2 (5 nM) and/or EGF (18 nM), and Luciferase assays were performed 18 h after addition of growth factor.

Received 2 June; accepted 18 August 1997.

1. Roberts, A. B., Sporn, M. B. in *Peptide Growth Factors and their Receptors* (eds Sporn, M. B. & Roberts, A. B.) 419–472 (Springer, Heidelberg, 1990).
2. Hogan, B. L. M. Bone morphogenetic proteins: multifunctional regulators of vertebrate development. *Genes Dev.* **10,** 1580–1594 (1996).
3. Eppert, K. *et al.* MADR2 maps to 18q21 and encodes a TGFβ-regulated MAD-related protein that is functionally mutated in colorectal carcinoma. *Cell* **86,** 543–552 (1996).
4. Graff, J. M., Bansal, A. & Melton, D. A. *Xenopus* Mad proteins transduce distinct subsets of signals for the TGFβ superfamily. *Cell* **85,** 479–487 (1996).
5. Hoodless, P. A. *et al.* MADR1, a MAD-related protein that functions in BMP2 signalling pathways. *Cell* **85,** 489–500 (1996).
6. Kretzschmar, M., Liu, F., Hata, A., Doody, J. & Massagué, J. The TGFβ family mediator Smad1 is phosphorylated directly and activated functionally by the BMP receptor kinase. *Genes Dev.* **11,** 984–995 (1997).
7. Lagna, G., Hata, A., Hemmati-Brivanlou, A. & Massagué, J. Partnership between DPC4 and SMAD proteins in TGF-β signalling pathways. *Nature* **383,** 832–836 (1996).
8. Lechleider, R. J., de Caestecker, M. P., Dehejia, A., Polymeropoulos, M. H. & Roberts, A. B. Serine phosphorylation, chromosomal localization and TGFβ signal transduction by human *bsp-1*. *J. Biol. Chem.* **271,** 17617–17620 (1996).
9. Macias-Silva, M. *et al.* MADR2 is a substrate of the TGFβ receptor and phosphorylation is required for nuclear accumulation and signaling. *Cell* **87,** 1215–1224 (1996).
10. Yingling, J. M. *et al.* Mammalian Dwarfins are phosphorylated in response to TGF-β and are implicated in control of cell growth. *Proc. Natl Acad. Sci. USA* **93,** 8940–8944 (1996).
11. Baker, J. & Harland, R. M. A novel mesoderm inducer, mMadr-2, functions in the activin signal transduction pathway. *Genes Dev.* **10,** 1880–1889 (1996).
12. Liu, F. *et al.* A human Mad protein acting as a BMP-regulated transcriptional activator. *Nature* **381,** 620–623 (1996).
13. Chen, X., Rubock, M. J. & Whitman, M. A transcriptional partner of MAD proteins in TGF-β signalling. *Nature* **383,** 691–696 (1996).
14. Niswander, L. & Martin, G. R. FGF-4 and BMP-2 have opposite effects on limb growth. *Nature* **361,** 68–71 (1993).
15. Ganan, Y., Macias, D., Duterque-Coquillaud, M., Ros, M. A. & Hurle, J. M. Role of TGFβs and BMPs as signals controlling the position of the digits and the areas of interdigital cell death in the developing chick limb autopod. *Development* **122,** 2349–2357 (1996).
16. Bernier, S. M. & Goltzman, D. Effect of protein and steroidal osteotropic agents on differentiation and epidermal growth factor-mediated growth of the CFK1 osseous cell line. *J. Cell Physiol.* **152,** 317–327 (1992).
17. Neubüser, A., Peters, H., Balling, R. & Martin, G. R. Antagonistic interactions between FGF and BMP signaling pathways: a mechanism for positioning the sites of tooth formation. *Cell* **90,** 247–255 (1997).
18. Hata, A., Lo, R. S., Wotton, D., Lagna, G. & Massagué, J. Mutations increasing autoinhibition inactivate tumour suppressors Smad2 and Smad4. *Nature* **388,** 82–87 (1997).
19. Kim, J., Johnson, K., Chen, H. J., Carroll, S. & Laughon, A. *Drosophila* Mad binds to DNA and directly mediates activation of *vestigial* by Decapentaplegic. *Nature* **388,** 304–308 (1997).
20. Zhang, Y., Feng, X.-H, Wu, R.-Y. & Derynck, R. Receptor-associated Mad homologues synergize as effectors of the TGF-β response. *Nature* **383,** 168–172 (1996).
21. Wu, R.-Y., Zhang, Y., Feng, X.-H. & Derynck, R. Heteromeric and homomeric interactions correlate with signalling activity and functional cooperativity of Smad3 and Smad4/DPC4. *Mol. Cell. Biol.* **17,** 2521–2528 (1997).
22. Hahn, S. A. *et al. DPC4*, a candidate tumor suppressor gene at human chromosome 18q21.1. *Science* **271,** 350–353 (1996).
23. Shi, Y., Hata, A., Lo, R. S., Massagué, J. & Pavletich, N. P. A structural basis for mutational inactivation of the tumour suppressor Smad4. *Nature* **388,** 87–93 (1997).
24. Zhang, Y., Musci, T. & Derynck, R. The tumor suppressor Smad4/DPC4 as a central mediator of Smad function. *Curr. Biol.* **7,** 270–276 (1997).
25. Hill, C. S. & Treisman, R. Transcriptional regulation by extracellular signals: mechanisms and specificity. *Cell* **80,** 199–211 (1995).
26. Vandenabeele, P., Declercg, W., Beyaert, R. & Fiers, W. Two tumour necrosis factor receptors: structure and function. *Trends Cell Biol.* **5,** 392–399 (1995).
27. Marshall, C. J. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell* **80,** 179–185 (1995).
28. Franke, T. F., Kaplan, D. R. & Cantley, L. C. PI3K: downstream AKTion blocks apoptosis. *Cell* **88,** 435–437 (1997).
29. Besser, D. *et al.* Regulation of the urokinase-type plasminogen activator gene by the oncogene Tpr-Met involves GRB2. *Oncogene* **14,** 705–711 (1997).
30. Blenis, J. Signal transduction via the MAP kinases: proceed at your own RSK. *Proc. Natl Acad. Sci. USA* **90,** 5889–5892 (1993).
31. ten Dijke, P. *et al.* Identification of type I receptors for Osteogenic Protein-1 and Bone Morphogenetic Protein-4. *J. Biol. Chem.* **269,** 16985–16988 (1994).
32. Schagger, H. & von Jagow, G. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **166,** 368–379 (1987).
33. Gu, W., Shi, X.-L. & Roeder, R. G. Synergistic activation of transcription by CBP and p53. *Nature* **387,** 819–823 (1997).
34. Massagué, J., Hata, A. & Liu, F. TGF-*β* signalling through the Smad pathway. *Trends Cell Biol.* **7,** 187–192 (1997).

Acknowledgements. We thank the Genetics Institute for providing BMP; Memorial Sloan-Kettering Cancer Center for use of the Molecular Cytology Core Facility; and I. Timokhina for discussions. This work was supported by NIH grants to J.M. and to Memorial Sloan-Kettering Cancer Center. M.K. is a recipient of a postdoctoral fellowship from the Deutsche Forschungsgemeinschaft. J.M. is an investigator of the Howard Hughes Medical Institute.

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Smad6 inhibits signalling by the TGF-β superfamily

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SMAD proteins[1] have been identified as signalling mediators of the TGF-β superfamily, which is involved in a range of biological activities including cell growth, morphogenesis, development and immune responses[2,3]. Smad1, Smad2, Smad3 and Smad5 are ligand-specific: Smad1 and Smad5 transduce signals from bone morphogenetic proteins[4–7], and Smad2 and Smad3 mediate signalling by TGF-β and activin[8,9], whereas Smad4 acts as a common signalling component[10]. For example, Smad2 is phosphorylated by the TGF-β type I receptor upon ligand binding, forms a heteromer with Smad4, and then translocates into the nucleus where it activates transcription[10,11]. Here we report the isolation of Smad6 in the mouse. Smad6 is quite different in structure from the other SMAD proteins, and forms stable associations with type I receptors. Smad6 interferes with the phosphorylation of Smad2 and the